

# Localization of Dengue Virus in Naturally Infected Human Tissues, by Immunohistochemistry and In Situ Hybridization

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Dengue viral antigens have been demonstrated in several types of naturally infected human tissues, but little is known of whether these same tissues have detectable viral RNA. We studied tissue specimens from patients with serologically or virologically confirmed dengue infections by immunohistochemistry (IHC) and in situ hybridization (ISH), to localize viral antigen and RNA, respectively. IHC was performed on specimens obtained from 5 autopsies and 24 biopsies and on 20 blood-clot samples. For ISH, antisense riboprobes to the dengue E gene were applied to tissue specimens in which IHC was positive. Viral antigens were demonstrated in Kupffer and sinusoidal endothelial cells of the liver; macrophages, multinucleated cells, and reactive lymphoid cells in the spleen; macrophages and vascular endothelium in the lung; kidney tubules; and monocytes and lymphocytes in blood-clot samples. Positive-strand viral RNA was detected in the same IHC-positive cells found in the spleen and blood-clot samples. The strong, positive ISH signal in these cells indicated a high copy number of viral RNA, suggesting replication.

Dengue infection is the most prevalent arthropod-borne viral disease in subtropical and tropical regions of the world [1]. The dengue virus (genus *flavivirus*, family *Flaviviridae*) consists of a single positive-strand RNA surrounded by an icosahedral nucleocapsid. There are 4 distinct serotypes of dengue virus [2]. Infection with 1 serotype provides lifelong protective immunity to that serotype, but there is no cross-protective immunity between serotypes [3]. Infection with dengue virus causes either a relatively mild disease, known as classic dengue fever (DF), or a more severe form, dengue hemorrhagic fever (DHF), a fulminating illness that is characterized by hemorrhagic manifestations and plasma leakage and that can progress to dengue shock syndrome (DSS) and death [4].

Studies of specimens obtained from patients with

DHF/DSS using immunofluorescence and immunohistochemistry (IHC) revealed the presence of viral antigens in many tissues, including liver, spleen, lymph node, thymus, kidney, lung, and skin, but mainly in mononuclear phagocytic cells [5–9]. The mere presence of viral antigens within cells, especially phagocytic cells, does not necessarily mean that the cells in question support viral replication, since antigens may represent phagocytized, killed virus or sequestered immune complexes in the process of being degraded. Evidence from in vitro studies suggested that other cells (e.g., hepatocytes, B and T lymphocytes, endothelial cells, and fibroblasts) could be potential targets for virus infection and replication, but relatively little is known about the involvement of these cells in in vivo infections [10–21].

The in situ hybridization (ISH) technique allows for the precise localization of dengue viral RNA in infected tissues. If the positive staining is intense, it could suggest that viral replication may have occurred in these tissues. Indeed, this technique has shown the presence of viral RNA in the thymus and peripheral lymphocytes, suggesting that these cells support in vivo viral replication [16]. However, these findings were based on only a few cases and a limited range of tissue types and, thus, need to be further investigated and confirmed. The

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purpose of the present study is to investigate the cellular localization of dengue virus in naturally infected human tissues by applying both the IHC technique and the ISH technique to tissue specimens obtained from patients with serologically or virologically confirmed dengue infection. From previous virologic surveillance studies, dengue serotypes 1, 2, and 3 were responsible for most outbreaks in Malaysia; therefore, the focus was on these 3 serotypes. [22].

## MATERIALS AND METHODS

### Control Materials

**Positive control tissue specimens.** Dengue 1 (Hawaii), dengue 2 (New Guinea C), and dengue 3 (H87) viruses were obtained from S. K. Lam (World Health Organization [WHO] Collaborating Centre for Arbovirus Reference and Research, Kuala Lumpur, Malaysia; Department of Medical Microbiology, University of Malaya). One-day-old suckling mice were injected intracerebrally with dengue 1 and 2. Sick mice were killed, and the brains were harvested and fixed in 10% buffered formalin for at least 48 h before routine processing and paraffin-embedding. Since it was difficult to infect mice with dengue 3, infected pig kidney cells (gift from Jane Cardosa, University of Malaysia Sarawak, Sarawak) were used as a positive control instead. Infected cells were harvested and resuspended in PBS and, to improve cell adherence, were spotted onto slides coated with 3-aminopropyltriethoxy-silane (Sigma). The slides were air-dried, wrapped in aluminum foil, and stored at  $-80^{\circ}\text{C}$ . Before use, the cells were fixed in either 4% paraformaldehyde or acetone for 20 min at  $4^{\circ}\text{C}$ .

**Negative control tissue specimens.** Negative controls comprised (1) uninfected suckling mouse brain, (2) suckling mouse brain experimentally infected with Japanese encephalitis virus (JEV), and (3) human brain or spinal cord naturally infected with either measles, JEV, or enterovirus 71. These tissue specimens were fixed in the same manner as were the positive controls, except for the human tissue specimens, which were fixed for  $>2$  weeks.

### Test Materials

All the cases in the present study were archived in the Department of Pathology, University of Malaya, Malaysia. Tissue specimens obtained from 5 autopsies and from 9 postmortem and 15 antemortem biopsies and 20 peripheral blood-clot samples from patients clinically diagnosed—mainly as having DF, DHE, or DSS—were examined (table 1). These patients were included in the study because they were positive by either dengue IgM serologic testing, viral culture, or reverse-transcription polymerase chain reaction (RT-PCR). In the case of antemortem biopsies, they were done as part of the clinical investiga-

tions performed before the diagnosis of dengue infection. All biopsies and autopsies were done after obtaining consent from the patient or the next of kin. Blood-clot samples from suspected cases of dengue infection were originally sent to the Department of Medical Microbiology, University of Malaya, for the purpose of dengue serologic testing.

Autopsy tissue specimens were fixed in 10% neutral buffered formalin for  $>1$  week, and biopsy specimens were fixed for at least 24 h, before routine processing and embedding. After serum separation by centrifugation, residual blood clots were formalin-fixed for at least 2 days, routinely processed, and paraffin-embedded as above. Four-micrometer-thick sections from tissue blocks were placed on silanized slides and dewaxed for 30–60 min at  $60^{\circ}\text{C}$ , followed by serial xylene and alcohol washes before IHC and ISH.

### IHC

Antidengue polyclonal antibodies (hyperimmune mouse ascitic fluid) for 3 serotypes were obtained from R. E. Shope (WHO Center for Tropical Diseases; University of Texas Medical Branch, Galveston). These antibodies were tested for specificity and possible cross-reactivity between dengue strains and other unrelated viruses, and IHC conditions were optimized before use.

The entire IHC procedure was performed at room temperature, except where stated otherwise. Deparaffinized tissue sections were washed in running water for 10 min and digested with 100  $\mu\text{g}/\text{mL}$  proteinase K (Promega) in a buffer (0.01 mol/L Tris-HCl [pH 7.8], 0.005 mol/L EDTA, and 0.5% SDS) for 15 min. After a second wash with water, the sequential incubations were (1) 3%  $\text{H}_2\text{O}_2$  in absolute methanol for 20 min; (2) normal rabbit serum (Gibco BRL) diluted 1:20 in Tris-buffered saline (TBS), for 20 min; (3) primary antibody (diluted 1:500 in TBS), overnight at  $4^{\circ}\text{C}$ ; (4) biotinylated rabbit anti-mouse antibody (diluted 1:300; Dako); (5) avidin biotin-horse-radish peroxidase complex (Dako) and 3,3' diaminobenzidine tetrahydrochloride (Sigma), according to the manufacturers' protocols. Between steps, the slides were washed in TBS for 5 min. Finally, the slides were washed in running water, counterstained in Harris hematoxylin, dehydrated, and mounted with DPX (Sigma). In addition to the negative controls, an adsorption control in which IHC was performed using dengue antibody after absorption with excess dengue virus antigens and a "minus primary antibody" control was also included in the experiments.

### Riboprobe Preparation

Dengue viral RNA was extracted from dengue 1-, 2-, and 3-infected C6/36 cells by use of TRIzol reagent (Gibco BRL), and respective cDNAs were produced by use of published specific primers to the E gene and the Access RT-PCR Kit (Promega) [23]. RT-PCR was performed in a thermocycler (DNA engine

**Table 1. Clinical diagnosis, laboratory findings, and tissue specimens examined in patients with dengue infection.**

Type of specimen, case number	Clinical diagnosis	Laboratory results	Tissue specimen(s) examined
<b>Autopsy</b>			
1	DSS	IgM positive	Multiple organs
2	Acute hemorrhagic pancreatitis	IgM negative, culture negative, RT-PCR positive for dengue 2 in serum	Multiple organs
3	DHF	IgM negative, dengue 1 isolated	Multiple organs
4	DSS and pulmonary hemorrhage	IgM positive	Multiple organs
5	DSS and diabetes mellitus	IgM positive	Multiple organs
<b>Postmortem biopsy</b>			
6	DHF	IgM positive	Liver
7	DHF	IgM positive	Liver
8	DHF	IgM positive	Liver, kidney, heart
9	DSS	IgM negative, dengue 3 isolated	Liver
10	DHF	IgM positive	Liver, lung
11	DSS	IgM positive	Liver, kidney
12	DHF	IgM positive	Liver
13	DSS	IgM negative, dengue 3 isolated	Liver
14	DSS and pulmonary hemorrhage	IgM positive	Liver, kidney, lung
<b>Antemortem biopsy</b>			
15	DSS	IgM positive	Liver
16	DF	IgM positive	Liver
17	DF	IgM positive	Liver
18	DHF	IgM positive	Liver
19	DHF	IgM positive	Liver
20	DHF with acute hepatitis	IgM positive	Liver
21	DF	IgM positive	Bone marrow
22	DF	IgM positive	Bone marrow
23	DHF	IgM positive	Bone marrow
24	DF	IgM positive	Bone marrow
25	DHF	IgM positive	Bone marrow
26	DF	IgM positive	Bone marrow
27	DF	IgM positive	Bone marrow
28	DF	IgM positive	Bone marrow
29	DF	IgM positive	Bone marrow
<b>Peripheral blood</b>			
30–49	All DF	All IgM positive	All blood clots

**NOTE.** DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; RT-PCR, reverse-transcription polymerase chain reaction.

PTC-200 Peltier; MJ Research), and the conditions were the following: (1) 45 min at 48°C then 1 min at 94°C; (2) 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, for 40 cycles; and (3) 5 min at 72°C.

The ~260-bp PCR fragments were purified by use of the High Pure PCR Purification Kit (Boehringer Mannheim), cloned into a suitable vector (pGEMT-Easy; Promega), and transformed into competent *Escherichia coli* cells (JM109; Promega). White and

pale blue colonies were identified on X-Gal minimal agar plates and screened for the presence and orientation of the inserts by *EcoRI* restriction endonuclease digestion and by use of PCR using T7 and dengue-specific primers. Plasmids with inserts in 2 orientations were isolated by use of a modified alkaline lysis method [24]. After linearization with *SpeI* restriction endonuclease (Promega), antisense and sense riboprobes were generated by use of the DIG RNA Labeling Kit SP6/T7 (Boehringer Mannheim).

**Table 2. Naturally-infected human tissue specimens and cells positive for dengue virus by immunohistochemistry and in situ hybridization.**

Tissue/organ	No. of cases			Positive cells		Case number(s) <sup>a</sup>
	Tested	IHC positive	ISH positive	IHC	ISH	
Liver	20	3	0	Kupffer cell, endothelium, lymphocytes and monocytes in lumen of blood vessels	Nil	3, 10, 11
Spleen	5	3	2	Macrophages, binucleated and multinucleated giant cells; lymphoid, centroblastic-like, immunoblast-like cells	Macrophages, binucleated cells; reactive lymphoid, centroblast-like, immunoblast-like cells	1, 3, 4
Kidney	8	3	0	Tubules	Nil	3, 4, 11
Lung	6	1	0	Alveolar macrophages, endothelium, monocytes in lumen of blood vessels	Nil	4
Bone marrow	9	1	0	Myeloid cells	Nil	25
Blood clot	20	8	7	Monocytes, lymphocytes	Monocytes, lymphocytes	36–43

**NOTE.** IHC, immunohistochemistry; ISH, in situ hybridization; Nil, no positive-staining cells.

<sup>a</sup> As in table 1.

Digoxigenin (DIG)-labeled riboprobes were purified as above and stored at  $-80^{\circ}\text{C}$  until use.

### ISH

We used the ISH method described by Killen et al. [16]. The sensitivity and specificity of the riboprobes were tested and optimized by varying pretreatment with proteinase K, before ISH was performed on all IHC-positive, and some IHC-negative, tissue specimens.

In brief, deparaffinized tissue sections were pretreated with HCl and proteinase K (100  $\mu\text{g}/\text{mL}$ ; 15 min;  $37^{\circ}\text{C}$ ). The hybridization and posthybridization steps were performed as described elsewhere [16]. Approximately 0.5–1.0 ng of either antisense or sense riboprobes (used as a mixture of 3 serotype-specific probes or as single probes) was pipetted onto tissue sections, covered with diethylpyrocarbonate-treated coverslips, and incubated for 16–20 h at  $45^{\circ}\text{C}$ . Probes that had hybridized with viral RNA were detected by use of an anti-DIG antibody conjugated to alkaline phosphatase and nitroblue tetrazolium/5-bromo-4-chloro-indol-3-indolyl phosphate color substrate (Boehringer Mannheim). The slides were counterstained with Mayer's hematoxylin and aqueous-mounted with glycergel (Dako).

### RESULTS

**Antibody specificity and sensitivity.** Dengue antibodies to all 3 serotypes cross-reacted with each other. Dengue 1 antibody showed the strongest staining on positive controls and thus was used for all subsequent IHC assays. Specificity testing of this antibody against JEV, enterovirus 71, and measles virus-infected tissue specimens convincingly showed it to be nonreactive for these viruses. All the other negative controls were also found to be negative.

**IHC findings.** Dengue viral antigens were detected in the liver, spleen, lung, kidney, and peripheral blood leukocytes

(PBLs) (table 2). Other tissue specimens, including thymus, lymph node, thyroid, pancreas, heart, adrenal gland, skeletal muscle, intestine, and brain, were found to be negative. Megakaryocytes in the bone marrow were consistently found to be negative for viral antigens.

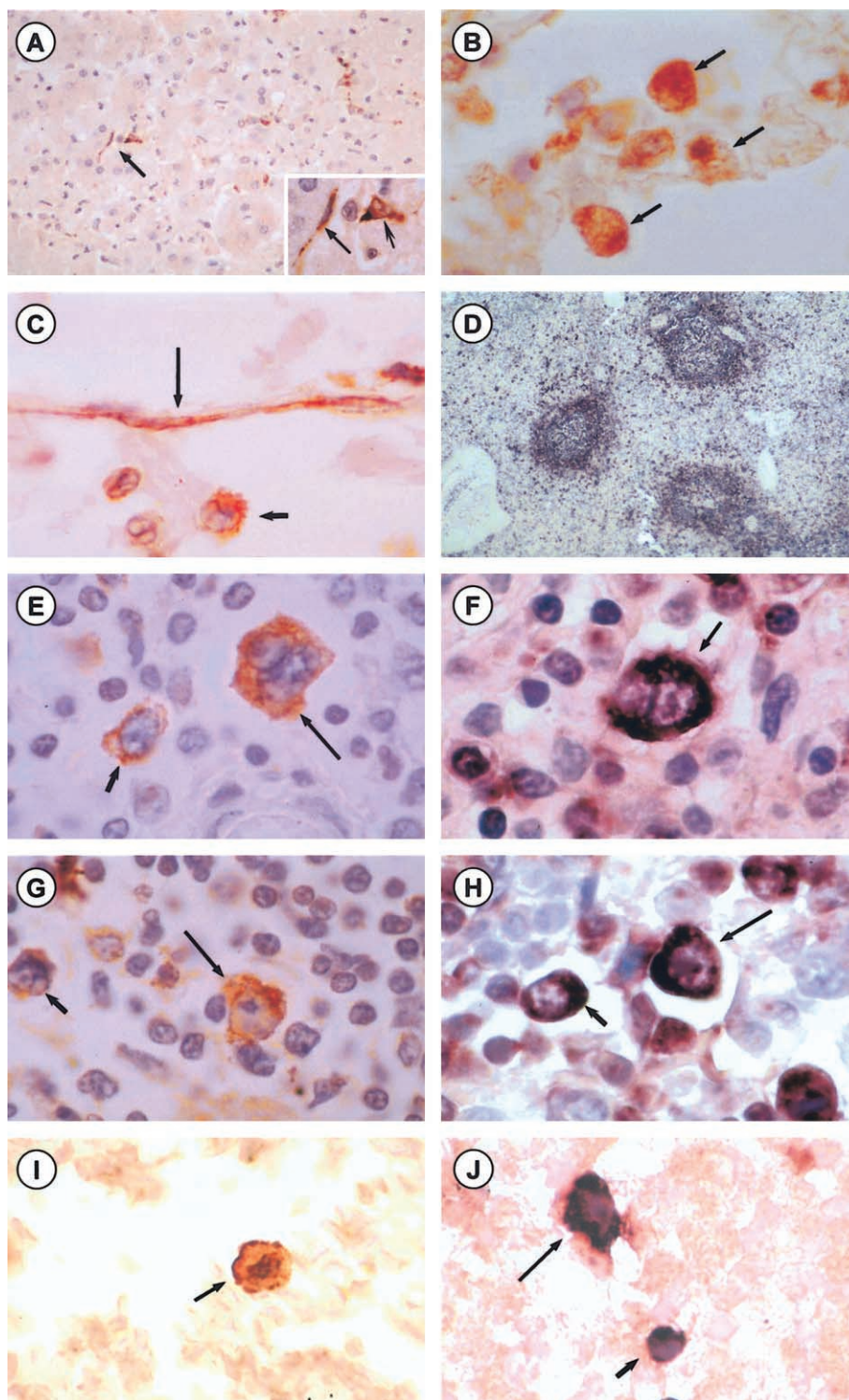
In general, antigens were immunolocalized to the cytoplasm, with no nuclear staining whatsoever. In the liver, fine granular deposits of dengue viral antigens were localized predominantly within Kupffer cells, endothelium (figure 1A), and peripheral monocytes in vascular lumen. In the spleen, viral antigens were clearly observed in the cytoplasm of macrophages, binucleated cells (figure 1E), multinucleated giant cells, and reactive lymphoid cells in the red pulp. In the white pulp, lymphoid cells, and some centroblast-like and immunoblast-like cells (figure 1G) in the germinal centers of lymphoid follicles were found to be IHC positive. In the kidney, viral antigens were detected as discrete granular deposits within the lining cells of the tubules. Viral antigens were detected in alveolar macrophages (figure 1B), pulmonary vascular endothelium, and monocytes inside blood vessels in the lung (figure 1C).

The majority of PBLs found to be positive for viral antigens were monocytes (figure 1I), although some lymphocytes were also found to be positive. Seven of the 8 positive blood samples were collected between days 4 and 8 of fever, and 1 was collected at day 15 of fever.

**Riboprobe specificity and sensitivity.** DIG-labeled antisense riboprobes used in optimized ISH assays could detect dengue viral RNA in positive control tissue specimens, giving clear signals with little background staining. Respective riboprobes were also serotype specific. Moreover, specificity tests showed that there was no evidence of cross-hybridization with JEV, enterovirus 71, or measles RNA. Uninfected tissue and blood samples showed negative staining.

**ISH findings.** In the splenic red pulp, viral RNA was localized to the cytoplasm of macrophages, reactive lymphoid,





**Figure 1.** A, Dengue viral antigens detected in the liver in Kupffer and endothelial cells (arrow), but not in hepatocytes. *Inset*, A Kupffer cell (short arrow) and an endothelial cell (long arrow). Shown are dengue viral antigens in the lung in alveolar macrophages (B, arrows), vascular lumen monocyte (C, short arrow), and vascular endothelium (C, long arrow). D, Dengue viral RNA localized in macrophages and reactive lymphoid cells in the red pulp and white pulp of the spleen. Shown are dengue viral antigens and RNA in the binucleated cells (E, long arrow, and F, arrow, respectively) in the red pulp, and viral antigens in a macrophage (E, short arrow, and G, short arrow). G, Viral antigens localized to an immunoblast-like cell (arrow). H, Dengue viral RNA in a centroblast-like cell (short arrow) and immunoblast-like cell (long arrow) in the germinal centers. I, Dengue viral antigens in a peripheral blood monocyte (arrow). J, Viral RNA in a peripheral monocyte (long arrow) and a lymphocyte (short arrow). A–C, E, G, and I, Immunohistochemistry, hematoxylin counterstain; D, F, H, and J, In situ hybridization, hematoxylin counterstain. Magnification,  $\times 100$  (A and D) and  $\times 500$  (B, C, and E–J).

binucleated cells (figure 1*F*), and multinucleated giant cells (table 2). In the white pulp, viral RNA was detected in centroblast-like and immunoblast-like cells (figure 1*D* and 1*H*) found in germinal centers. On occasion, there were also ISH-positive mononuclear cells in the lumen of splenic blood vessels. In the blood-clot samples, viral RNA was mainly localized to the cytoplasm of monocytes and some lymphocytes (figure 1*J*).

Repeat ISH using single serotype probes on the positive spleens showed evidence of dengue 1 in 1 case and dengue 3 in the other. For the blood-clot samples, 6 showed dengue 3 infection, and 1 showed dengue 1 infection. ISH using sense riboprobes did not detect any negative-strand RNA of replicative intermediates (RIs), in both test and positive controls. Liver Kupffer cells, lung alveolar macrophages, kidney tubules, and endothelium in these organs were found to be negative for viral RNA.

## DISCUSSION

Dengue viral antigens have been detected in various human tissues in previous studies, but it is not certain whether viral RNA can be localized to these same tissues, since little work has been done to establish this [5–9]. In fact, there are only 2 other published studies that have attempted to detect dengue viral RNA in a small number of naturally infected human tissues [16, 25].

The demonstration of positive-strand viral RNA by ISH, in reactive B lymphoid cells in the germinal centers of the spleen—including centroblasts and immunoblasts—and peripheral lymphocytes has not been reported previously. The mere presence of viral RNA does not necessarily imply that active intracellular viral replication has occurred. Nonetheless, a strong signal, indicating a high copy number, is probably good supportive evidence for infection and viral replication, particularly in cells that are not known to be phagocytic. This finding is supported by *in vitro* studies, which have demonstrated dengue virus replication in human B lymphoblastoid cells [11].

Unfortunately, in the present study, sense riboprobes, which theoretically should hybridize with RIs, were apparently negative on the same cells that were positive with antisense riboprobes. We are uncertain why this was so. Demonstration of RIs would have confirmed viral replication [16]. In recent studies using sensitive quantitative RT-PCR-based methods, RIs could be detected in cell cultures, as well as PBLs of patients with dengue infection, but the copy numbers were generally very low [26, 27]. In PBLs, for example, copy numbers ranged from only 40 to 11,000 copies/10<sup>6</sup> cells [27]. Furthermore, electron microscopic ISH studies of dengue 2-infected mosquito cells showed no hybridization signal, by use of the sense, single-stranded DNA probe, but showed a positive signal, by use of the antisense probe [28]. There is also the possibility that whatever RIs that were present in the tissue specimens could have

been destroyed as a result of formalin-fixation and tissue processing. All these possibilities could explain the apparent absence of RIs in the tissue specimens we tested, despite the strong positivity for positive-strand viral RNA. It may be possible to detect RIs using *in situ* PCR in formalin-fixed tissue specimens.

The peripheral blood lymphocytes found to be positive for viral antigen and RNA in the present study could represent either B or T cells. Dengue virus has been isolated from, and viral antigen has been demonstrated in, circulating B lymphocytes in both primary and secondary infections [29]. Furthermore, *in vitro* replication of dengue virus in mitogen-stimulated and -unstimulated human peripheral blood B lymphocytes has been reported elsewhere [11, 12]. Human primary B lymphocytes infected *in vitro* have recently been shown to produce interleukin-6 and tumor necrosis factor- $\alpha$ , suggesting that B lymphocytes may play an important role in dengue pathogenesis [12]. The permissiveness of T lymphocytes to dengue virus has been demonstrated in *in vitro* infections but not in *in vivo* infections [13, 14].

In our study, we have demonstrated for the first time the cellular localization of dengue viral RNA in splenic macrophages and peripheral blood monocytes. Again, the strong staining obtained suggested that viral replication may have occurred in these cells. Dengue RNA has been demonstrated before in macrophages in the lymph node and skin by use of an *in situ* PCR method [25]. In *in vitro* experiments, human peripheral blood monocytes were found to support viral replication [14, 30–32].

In contrast, the failure to detect viral RNA in antigen-positive Kupffer cells and alveolar macrophages suggests viral phagocytosis or immune complex sequestration rather than viral replication. Interestingly, *in vitro* dengue virus infection of human Kupffer cells showed that these cells were not involved in productive infection [33]. It is possible that, *in vivo*, Kupffer cells and alveolar macrophages do not support viral replication but may play a protective role against dengue virus by their phagocytic function. In the present study, the detection of viral antigens (with or without viral RNA) in cells of the mononuclear phagocytic system confirmed previous findings [5–9]. This supports the theory that monocytes and macrophages play a significant role in the pathogenesis of DHF/DSS, in view of their possible role in the infection-enhancement phenomenon, especially in secondary infection [34, 35]. It has been postulated that infected monocytes/macrophages and activated T lymphocytes release mediators, which act directly on the vascular endothelium to increase vascular permeability, resulting in plasma leakage, shock, and death [36–40].

Direct vascular endothelium infection may also contribute to DSS. Several *in vitro* studies have shown that viral replication occurs in endothelial cell cultures [15–20]. However, the present study has found no evidence of viral RNA in the endothelium of the organs studied, although viral antigens were

demonstrated in vascular endothelium in the lung and liver. Viral antigens have also been observed in vascular endothelium in the liver and, apparently, also in the brain [6, 7]. However, viral RNA or endothelial damage apparently has not been observed in natural human infections [36, 41]. The process of pinocytosis could explain the detection of viral antigens in vascular endothelium, since an ultrastructurally increased number of vacuoles and pinocytotic vesicles was observed in the endothelium of skin biopsy specimens from patients with DHF [41].

The absence of viral RNA in kidney tubular cells that immunolocalize for viral antigen suggests that viral replication did not occur in these cells. These antigens could represent reabsorbed immune complexes after elimination through the kidney, analogous to that in yellow fever, in which tubular cells also demonstrated the presence of yellow fever viral antigen [42]. In separate studies, viral antigens [5] and viral RNA have been demonstrated in hepatocytes by an in situ PCR method [25]. No viral antigens or RNA were detected in hepatocytes from our cases. The reasons for this may be that viral antigens seem to appear early in hepatocytes after infection, and production of infectious particles is rather low [43]. It is interesting that there seems to be no evidence for the involvement of megakaryocytes found in the bone marrow. This suggests that thrombocytopenia, a characteristic of DHF/DSS, may not be associated with failure of platelet production. There appears to be no relationship between detection of dengue virus and the severity of clinical disease (table 2). Nonetheless, more cases have to be studied to confirm this.

In conclusion, macrophages, peripheral blood monocytes, reactive splenic lymphoid cells, and peripheral lymphocytes may be the major target cells of dengue viral replication in natural human infections. These findings have to be confirmed by examining tissue specimens from more cases of human infection. Electron microscopy and in situ PCR could be useful tools to confirm viral replication.

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